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A comparison of the pupariation acceleration activity of pyrokinin-like peptides native to the flesh fly: Which peptide represents the primary pupariation factor?

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ABSTRACT

Five native pyrokinin-like peptides (Neb-PK-1, Neb-PK-2, Neb-PVK-1, [L^9]Neb-PVK-2, [I^9]Neb-PVK-2) identified in the neuropeptidome of the flesh fly *Neobellieria bullata* were compared for their quantitative and/or qualitative effects on puparium formation (pupariation). In a standard pupariation bioassay, both Neb-PVK-1 and [I^9]Neb-PVK-2 proved inactive, whereas [L^9]Neb-PVK-2 demonstrated only weak activity. In contrast, both Neb-PK-1 and Neb-PK-2 demonstrated potent threshold doses, with Neb-PK-2 about 10-fold more active than Neb-PK-1. Analysis of neuromuscular activity during pupariation using a tensiometric technique demonstrates that the two native Neb-PKs accelerate the onset of immobilization and cuticular shrinkage more than motor programs associated with retraction of the anterior segments and longitudinal body contraction. It was further determined that the sensitivity of various components of the pupariation process to these peptides decreases in the following order: immobilization > cuticular shrinkage > motor program for anterior retraction > motor program for longitudinal contraction ≅ tanning of cuticle of the newly formed puparium. A paradoxical situation was observed whereby the motor programs of pupariation are temporally dissociated from actual morphogenesis of the puparium. The tensiometric data suggest that the most likely candidate for a primary pupariation factor is Neb-PK-2, rather than Neb-PK-1.

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1. Introduction

Pupariation in cyclorrhaphous flies is a process of transformation of the soft integument of a wandering larva into a hard ovoid case called the puparium. It involves both behavioral activities (immobilization, retraction of the anterior segments, longitudinal body contraction), and sclerotization of the cuticle (deplasticization and hardening by phenolic tanning) that fixes the achieved morphological changes [15,19]. Pupariation can be accelerated by factors in extracts of various neural and neurohaemal organs, as well as in the haemolymph of pupariating larvae [14]. It has also been reported that leucopyrokinin (LPK), a member of the pyrokinin (FXPRLamide) class of neuropeptides isolated from the cockroach *Leucophaea maderae* [3], can also elicit acceleration of pupariation at low threshold doses [7,16,17]. It was not until 6 years after this initial observation that the first sequences of pyrokinin-like peptides native to the flesh fly were identified. An analysis of preparations of the perisympathetic organs of the flesh fly, *Neobellieria bullata*, led to the identification of a

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pyrokinin (Neb-PK-1) and two other related neuropeptides, Neb-PVK/CAP2b-1 and Neb-PVK/CAP2b-2, via mass spectrometric techniques [8]. All three are products of the capa gene. The sequence of Neb-PK-1 was reported as AGPSATTGVWFGPRLamide, although the mass spectrometric techniques employed could not distinguish between the isomers Leu and Ile in the C-terminal position. Nonetheless, all pyrokinins isolated and identified from insects have contained a C-terminal Leu; and we have made the assumption that conservation of sequence applies in this case as well. Neb-PK-1 shares the C-terminal octapeptide sequence GXWFGPRLamide with the PK-1 (Drm-PK-1) of *Drosophila* (X = V for *Neobellieria* and X = L for *Drosophila*, a conservative change). The other two peptides are members of the periviscerokinin(PVK)/cardioacceleratory peptide (CAP2b) peptide class, which shares some sequence similarity to the pyrokinins. The sequences of Neb-PVK/CAP2b-1 and Neb-PVK/CAP2b-2 were reported as NGGTSGLFAPRVamide and AGLIVYPRL/Iamide, respectively [8,6]. Subsequently, a second pyrokinin, designated Neb-PK-2, was isolated, purified and identified as SVQFKPRLamide. This differs from the PK-2 of *Drosophila* (Drm-PK-2) by only a P/Q substitution in position 3, and is a product of the hugin gene [10]. Strong immunoreactivity in the ring gland of wandering larvae with an antiserum against Drm-PK-2 suggested that the peptide is stored and/or released from the ring gland and serves as a pupariation factor, a presumed neurosecretory hormone regulating formation of the fly puparium [2]. This idea was supported by a very low threshold dose for acceleration of pupariation in the classic bioassay. Neb-PK-1, however, had not as yet been tried in the pupariation bioassay.

In the present paper, we evaluate the activity of capa-gene products (including Neb-PK-1) native to the flesh fly in the classical pupariation bioassay. In addition, we attempt to supplement the lack of information on the biological activity of the two native pyrokinins of the flesh fly *N. bullata* by performing a comparative analysis of the effects of synthetic preparations of both peptides on pupariation of flesh fly larvae using a combination of the classic pupariation bioassay with a tensiometric technique designed to record and analyze details of motor patterns of behavior and biomechanical changes in larval cuticle associated with formation of the barrel-shaped puparium.

2. Materials and methods

2.1. Experimental flies

Larvae of the fleshfly, *N. bullata* were reared by 200–300 specimens per batch on beef liver in small open disposable packets made from aluminium foil as described [13]. Fully-grown larvae that left the food were allowed to wander in dry sawdust until the first puparia appeared 36–40 h later. Then the batch was ready for collecting red spiracle (RS) stage larvae that can be distinguished by precocious tanning of the cuticle in the region of hind spiracles (peritreme). For the bioassay or tensiometric recordings early-RS larvae (2–3 h before pupariation) were used, unless indicated otherwise. At the end of the RS-stage the larva stops crawling and irreversibly retracts the first three front segments with the cephalopharyngeal apparatus ('the mouth hooks') (reteraction, R). Then the larva longitudinally contracts to the barrel shaped puparium (contraction, C) and its surface gets smooth by shrinking of the cuticle, eventually attaining the shape of the 'white puparium' (WP). Some 50–60 min after C the WP starts to change colour by phenolic tanning of the cuticle (T) and turns to an 'orange puparium'.

2.2. Classical bioassay for acceleration of pupariation

The test was performed as described by Žd'árek [13]. Briefly, the tested material was injected into early-RS larvae previously immobilised by chilling on ice. Control larvae were injected with solvent only. After removal from ice the injected larvae were kept in petri dishes lined with filter paper at 25 °C, and the time of R, C and T was recorded. The effects of a tested compound were expressed as a difference between the mean time, after which C and T occurred in the control and experimental larvae. Eight to 12 larvae were injected in each group and the test was twice or thrice repeated. Larvae were injected by means of a calibrated disposable glass capillary with pointed tip. Volume of injected solutions was 0.5–1 μL

Usually one of the injected larvae was taken for tensiometric measurements that were performed simultaneously with the bioassay in order to compare the occurrence of motor patterns of pupariation behaviour of tensiometrically recorded larva with visible signs of pupariation of the sham injected controls.

2.3. Peptide synthesis

Neb-PK-1 (AGPSATTGVWFGPRLa), Neb-PK-2(SVQFKPRLa), Neb-PVK/CAP2b-1 (NGGTSGLFAFPRVa), [L^9]Neb-PVK/CAP2b-2 (AGLIVPRLa), and [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa) were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [5]. Crude products were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 μm particle size, 100 A pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 mL/min. Delta-Pak C-18 retention times: Neb-PK-1 (AGPSATTGVWFGPRLa), 12.0 min; Neb-PK-2 (SVQFKPRLa), 6.0 min; Neb-PVK/CAP2b-1(NGGTSGLFAFPRVa), 12.25 min; [L^9]Neb-PVK/CAP2b-2 (AGLIVPRLa), 10.5 min; and [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa), 8.0 min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). Conditions: flow rate: 2.0 ml/min; isocratic with Solvent = 80% acetonitrile made to 0.01% TFA. WatPro retention times: Neb-PK-1 (AGPSATTGVWFGPRLa), 9.0 min; Neb-PK-2 (SVQFKPRLa), 8.75 min; Neb-PVK/CAP2b-1 (NGGTSGLFAFPRVa), 9.0 min; [L^9]Neb-PVK/

CAP2b-2 (AGLIVPRLa), 6.25 min; and [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa), 7.5 min. Amino acid analysis was carried out under previously reported conditions [5] and used to quantify the peptide and to confirm identity, leading to the following analyses: Neb-PK-1 (AGPSATTGVWFGPRLa), A[1.9], F[1.0], G[2.8], L[1.0], P[1.8], R[1.0], S[1.0], T[1.9], V[0.9]; Neb-PK-2 (SVQFKPRLa), A[1.0], F[1.0], G[2.7], L[2.0], P[1.1], R[1.0], S[0.9], V[1.1], Y[1.0]; Neb-PVK/CAP2b-1 (NGGTSGLFAFPRVa), F[1.0], K[1.1], L[1.0], P[1.0], Q[0.9], R[1.0], S[0.9], V[0.9]; [L^9]Neb-PVK/CAP2b-2 (AGLIVPRLa), A[1.0], G[0.9], I[0.8], L[2.0], P[1.0], R[1.0], V[0.9], Y[0.9]; and [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa), A[0.8], G[1.0], I[1.7], L[1.0], P[0.8], R[0.9], V[0.8], Y[1.1]. The identity of the peptide analogs were confirmed via MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Ltd., Manchester, UK) with the presence of the following molecular ions (M + H$^+$): Neb-PK-1 (AGPSATTGVWFGPRLa), 1516.1[M + H$^+$]; Neb-PK-2 (SVQFKPRLa), 973.6 [M + H$^+$]; Neb-PVK/CAP2b-1 (NGGTSGLFAFPRVa), 1322.1 [M + H$^+$]; [L^9]Neb-PVK/CAP2b-2 (AGLIVPRLa), 1000.7[M + H$^+$]; and [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa), 1000.8[M + H$^+$].

2.4. *Contact tensiometric measurements*

A non-invasive (contact) tensiometric technique of indirect detection of cuticular tension was used as previously described [18]. Changes of cuticular strain caused by both the muscular activity (rapid strain fluctuations) and shrinkage of the cuticle (slow rise of the baseline) were recorded via mechanical transduction of a strain gauge. The records served to analyze the effects of tested peptides on the motor programs of pupariation behaviour and changes of the properties of the cuticle. A larva to be measured was ventrally glued to a pinhead by instant adhesive (Loctite Corp., Dublin, Ireland). Against the pinhead a small metal disk was gently pressed to the dorsal surface of the larva by a week steel spring. Movements of the disk caused by changes of pressure in the hemocoel were picked up by a strain gauge attached to the spring. Sampling of changes of cuticular tension was done at a frequency of 5 Hz by an A/D converter Drak 4 (Papouch, Praha, CZ) and digitised data were visualized and analyzed by a PC using a RainbowReader software (Jiří Semecký, Praha, CZ). On a tensiogram, distinctive patterns of muscular contractions that characterise particular phases of pupariation behaviour (locomotion, immobilisation, retraction, contraction) are reflected in rapid reversible pulsations of hemocoelic pressure. Shrinkage and loss of plasticity of the sclerotizing cuticle cause a slow irreversible increase in the pressure baseline [18].

Using the method of tensiometric recording we were able to reveal direct effects of the tested peptides on neuromuscular activity and the cuticle. During normal pupariation four distinct patterns of motor activity can be distinguished [18]. Briefly, the muscles of a crawling or digging larva produce anteriorly directed body peristalsis that is recorded as a dense continuous train of rather regular complex pulses (Figs. 1 and 3, control). This locomotory pattern of the wandering phase is replaced with a pattern of an immobilization (I) phase, when the larva ceases locomotion. The muscular contractions continue but the direction of peristalsis is reversed. A train of contractions becomes interrupted with progressively extending pauses and interspaced with large singular peaks that reflect simultaneous contractions of the overall somatic musculature (Fig. 3b). During the later pauses the larva retracts the front segments, which is reflected by low regular spiking (Fig. 3c). We identify this period as the retraction (R) phase. Then the pulsation dramatically changes. The pauses disappear and the pulsation becomes composed of regular sharp peaks interspaced with broader multiple spike peaks. At the same time the baseline slowly rises indicating a steady increase of hemocoelic pressure due to shrinkage of the cuticle. During this contraction (C) phase the larva shortens into a white puparium and its cuticle becomes smooth. After about 20 min the broad multiple spike peaks disappear and the first sign of tanning (T) of the cuticle appears. Muscular activity gradually ceases after several more minutes.

At least 3 records for each dose of the tested compound were made and a representative one chosen for illustration. The tensiometric records began at a time when the larva recovered from chilling and resumed normal movements some 3–5 min after mounting on the tensiometer. All records used for illustration were adjusted to have identical time scales. For a detailed comparative analysis the time scales of the records were accordingly extended to identify patterns characteristic for the above described phases of pupariation (see Fig. 3 as an example). On the other hand, some records are presented with a greatly compressed time scale allowing for plain graphic expression of the rise of baseline tension, which reflects shrinkage of the cuticle (Fig. 2).

3. Results

3.1. *Flesh fly pupariation acceleration activity of native pyrokinin-like peptides*

In the classical pupariation acceleration bioassay using flesh fly larvae the native pyrokinin Neb-PK-1 (AGPSATTGVWFGPRLa) demonstrated relatively potent activity, with a threshold dose of 1 pm required to elicit a significant response (Table 1). The pupariation acceleration activity of the second pyrokinin, Neb-PK-2 (SVQFKPRLa), was previously reported to

Table 1 – Pupariation acceleration activity of native pyrokinin-like peptides on flesh fly (*N. bullata*) larvae

Peptide	Sequence	Pupariation acceleration [Threshold][a]
Neb-PK-1	AGPSATTGVWFG**PRL**a	1 pm
Neb-PK-2	SVQFK**PRL**a	0.1 pm[b]
Neb-PVK/CAP2b-1	NGGTSGLFAF**PRV**a	Inactive
[L^9]Neb-PVK/CAP2b-2	AGLIV**PRL**a[c]	50 pm
[I^9]Neb-PVK/CAP2b-2	AGLIV**PRI**a[c]	Inactive

[a] The values represent an average of 4–5 trials.
[b] Slightly less than the previously reported threshold dose of 0.2 pm [10].
[c] Neb-PVK/CAP2b-2 was synthesized as both the C-terminal L and I variants, as these two isomers could not be distinguished by the mass spectrometric methods employed to identify the sequence (Predel et al., 2003).

have an even more potent threshold dose of 0.2 pm. In our hands, we found the threshold dose of Neb-PK-2 to be slightly lower, at 0.1 pm. The Leu variant of Neb-PVK/CAP2b-2, [L^9]Neb-PVK/CAP2b-2 (AGLIVPRLa), demonstrated weak pupariation acceleration activity at a threshold dose of 50 pm. In contrast, the Ile variant of Neb-PVK/CAP2b-2, [I^9]Neb-PVK/CAP2b-2 (AGLIVPRIa), and Neb-PVK/CAP2b-1 (NGGTSGLFAFPRVa), proved completely inactive even up to a dose of 500 pm (Table 1).

3.2. *Effects of the active native pyrokinins of N. bullata on timing of pupariation events*

The two native peptides that were most active in the pupariation bioassay (Neb-PK-1 and Neb-PK-2) were further scrutinized by tensiometric analysis. The time of longitudinal contraction of the body (marked as C on tensiograms in Fig. 1; and indicated by an increase of the baseline in Fig. 2) was taken as a criterion to determine the extent of pupariation acceleration, Neb-PK-1 was inactive at a level of 0.5 pmol $larva^{-1}$ (Figs. 1c and 2c) while Neb-PK-2 showed some activity at a dose as low as 0.05 pmol $larva^{-1}$ (Figs. 1g and 2g). When a larva was injected with the lowest dose (0.05 pmol) of Neb-PK-2 very early in the RS-stage (3–4 h before expected C; Figs. 1h and 2h), the effect on cuticle shrinkage was less pronounced, if at all, than in a larva injected later (1–2 h before expected C; Figs. 1g and 2g). Thus, the age of a larva at the time of injection can affect the threshold dose required for acceleration of puparial contraction caused by shrinkage of the cuticle. Interestingly, when pupariation was accelerated by either of the tested peptides, retraction of the anterior segments (R) did not precede C, as it does normally. Actually, R was greatly delayed after C (Fig. 1) and the front of the larva was *completely* withdrawn only shortly before the motor pattern of the contraction phase (here designated ''C'', to distinguish it from C caused by cuticle shrinkage) began. Movements of the anterior retractor muscles can be recognized on tensiograms as low regular peaks occurring during pauses between the trains of major contractions at the end of immobilization/retraction phase (Fig. 3c). Tanning (T) of pyrokinin-injected specimens was also accelerated relative to T of the sham injected controls (marked as T on tensiograms in Fig. 1).

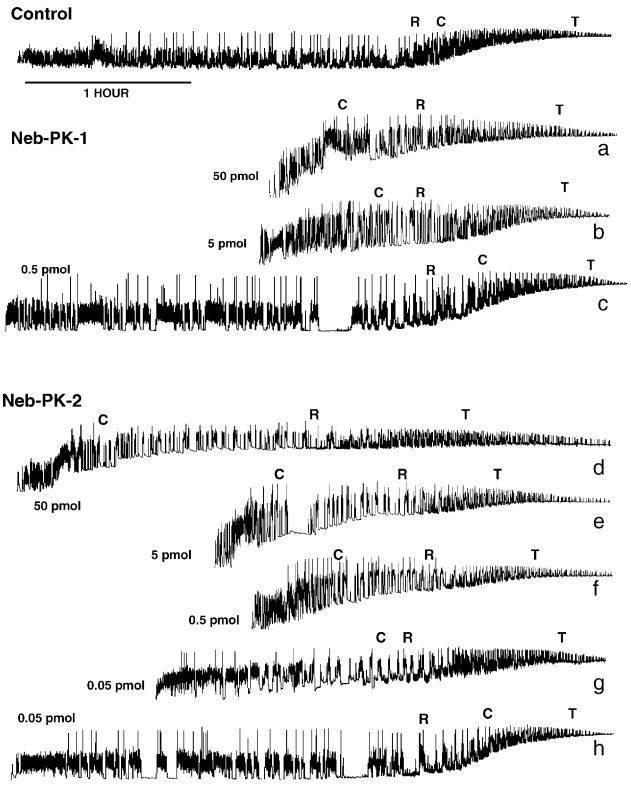

Fig. 1 – Tensiometric records of haemocoelic pulsations of *N. bullata* larvae injected with Ringer saline (control) and various doses of pyrokinins (Neb-PK-1; a–c and Neb-PK-2; d–h) 2–3 h before the expected onset of pupariation. The horizontal bar indicates a period of 1 hour and applies to all records. *R*, time of anterior retraction; *C*, time of longitudinal contraction; *T*, time of the onset of cuticular tanning.

3.3. *The effects of the native pyrokinins of N. bullata on behavioral patterns and structural changes of the cuticle during pupariation*

Using a tensiometric method we recorded patterns of hemocoelic pulsations from RS-larvae treated with different doses of both Neb-PK-1 and Neb-PK-2 and compared them with the patterns recorded from saline injected controls. Active doses of both tested peptides elicited effects on motor patterns of pupariation behaviour in a manner similar to other synthetic pyrokinins tested earlier [20]. The pattern of muscular peristalsis typical for crawling is precociously terminated and replaced by a prolonged period of patterns typical for the I/R phase (Fig. 1). Shortly after crawling movements ceased, the baseline starts to rise (Fig. 2). This causes longitudinal contraction (C) and leads to a smoother cuticular surface–typical attributes of the white puparium. Thus the WP is formed much earlier than the C pattern of muscular contractions would suggest. Since formation of the puparium in PK-injected larvae is temporally dissociated from a motor pattern, which is normally associated with longitudinal body contraction, we designate that ''functionless'' contraction pattern of PK-treated larvae as ''C'' to distinguish it from C, which refers to an actual change in morphology.

In further experiments, we tried to establish when the ''C'' pattern of PK-injected larvae occurs in relation to the occurrence of the analogous C pattern in controls. To this end we sham-injected cohorts of larvae of the same age as a tensiometrically gauged PK-injected larva and compared the mean times of their R (completion of R normally indicates beginning of C) with the beginning of the ''C'' pattern of the recorded specimen. Fig. 4 shows that the time of ''C'' was significantly accelerated in larvae treated with all tested doses of Neb-PK-2, while ''C'' of Neb-PK-1 treated larvae showed acceleration only at the highest (50 pmol $larva^{-1}$) tested dose.

Our tensiometric records also revealed that some low doses of PKs, which do not cause acceleration detectable by the classical pupariation bioassay, can still affect motor patterns of pupariation behaviour. Some larvae treated with 0.5 and 0.05 pmol $larva^{-1}$ of Neb-PK-1 and Neb-PK-2, respectively, retracted and contracted on a normal schedule, but their

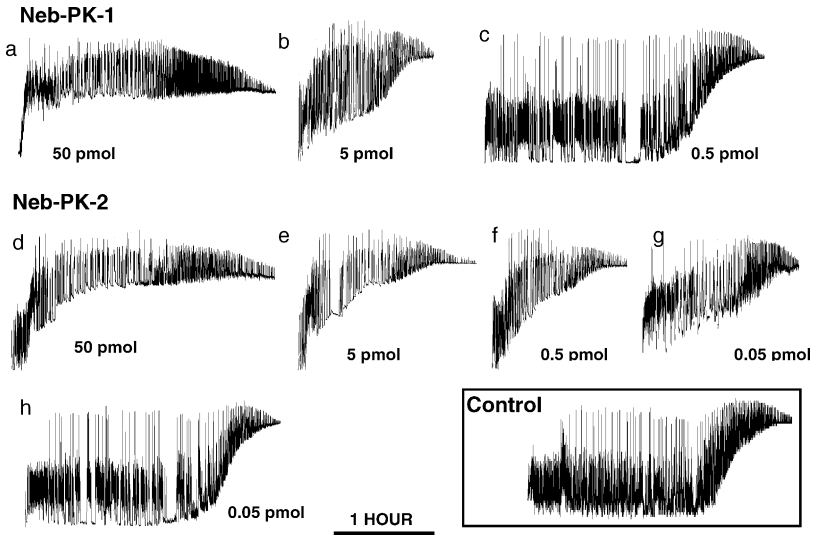

Fig. 2 – Tensiometric records of haemocoelic pulsations of *N. bullata* larvae injected with Ringer saline (control) and various doses of pyrokinins (Neb-PK-1; a–c, and Neb-PK-2; d–h) 2–3 h before the expected onset of pupariation. The horizontal bar indicates a period of 1 h and applies to all records. The records are made at a very slow chart speed to highlight the rise in the pressure baseline, reflecting the intrapuparial pressure produced by the shrinkage of the cuticle that precedes tanning and sclerotization.

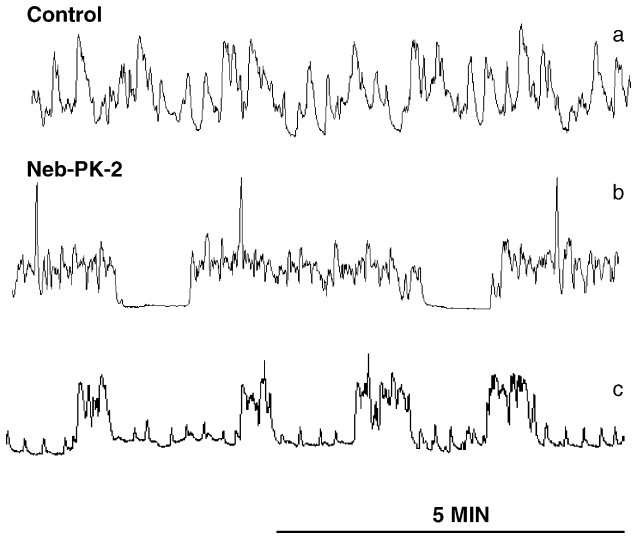

Fig. 3 – Tensiometric records of haemocoelic pulsations of *N. bullata* larvae injected with Ringer saline (control) and 0.05 pmol of Neb-PK-2 some 2–3 h before expected onset of pupariation. The records 'a' and 'b' were taken approximately 45 min after injection and show details of motor patterns characteristic for crawling (a) and an early immobilization/retraction stage (b) of pyrokinin accelerated pupariation. The record 'c' was taken approximately 1 h after injection of the same larva and shows details of motor patterns characteristic of a late immobilization/retraction stage, when retraction of the anterior segments was taking place. The movements of anterior retractor muscles are expressed as small regular peaks between the trains of major contractions. The horizontal bar indicates a period of 5 min and applies to all records.

immobilization stage was greatly extended at the expense of crawling (Figs. 1c and h and 3b and c). Tanning of the cuticle of PK-injected larvae regularly starts during the second half of the "C" pattern (Fig. 1), and is accelerated to a similar degree as the "C" pattern.

4. Discussion

The classical pupariation bioassay showed that only peptides having the—PRLa sequence in the C terminal region accelerate all aspects of pupariation. A periviscerokinin Neb-PVK/CAP2b-1, with a—PRVa at the C-terminus, proved to be inactive. This confirms our earlier findings that the presence of Leu at this position is critical for pupariation acceleration activity, and that steric bulk adjacent to the alpha carbon, as is present in Val or Ile, leads to loss of activity [7,20]. Peptides of the periviscerokinin/CAP2b family have been associated with stimulation of Malpighian tubule fluid secretion in the housefly and fruit fly [1,6], and it is quite possible that the true function of Neb-PVK/CAP2b-1 and -2 is the regulation of water and ion balance in the flesh fly.

In the present paper, we focused on the two most active native pyrokinins of the flesh fly *N. bullata* (Neb-PK-1 and Neb-PK-2) with the aim to ascertain which represents the primary pupariation factor of the flesh fly. Verleyen et al. [10] believe that this pyrokinin is the factor. They propose that Neb-PK-2 is released into the haemolymph from the neuroendocrine system of the CNS shortly before pupariation, as Fraenkel's group suggested more than 30 years ago [9]. This contention is supported by the fact that the in situ radioimmunoassay showed the greatest concentration of Neb-PK-2 in the corpora cardiaca region of the ring gland and the associated nerves. Our original finding that the activity of the other native pyrokinin isolated from the perisympathetic organs of the

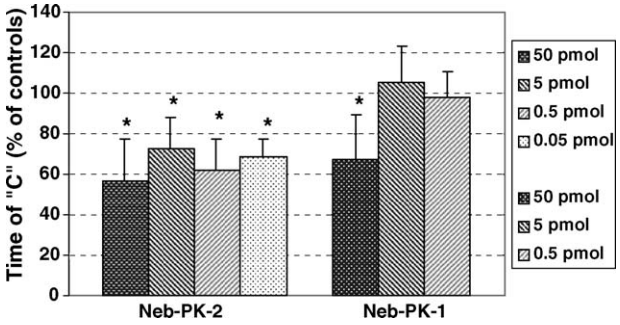

Fig. 4 – Time of "C" motor patterns (mean ± S.D.) of puparia accelerated with different doses of pyrokinins expressed as a percentage of the average time of anterior retraction of the sham-injected controls. Significant differences from the control ($p < 0.05$) are marked with an asterisk.

CNS, Neb-PK-1, is lower by a factor of 10 supports the idea that Neb-PK-1 may play only a minor role in the pupariation process and/or, indeed, have an entirely different function in the flesh fly.

The Verleyen group did not compare the activity of Neb-PK-2 with the other native pyrokinin (Neb-PK-1), unknown to them at the time of their study. Therefore, we undertook this study to fill this gap. By means of a sensitive tensiometric method, we compared qualitative as well as quantitative aspects of the action of the two peptides. We have found that the value of the lowest effective dose depends to a certain extent on the age of larvae at injection; younger larvae are less sensitive than older ones. Further, we showed that low doses of the two pyrokinins, which do not cause acceleration detectable in the classical pupariation bioassay, nevertheless accelerate the onset of the immobilization stage. The threshold dose of this effect for Neb-PK-2 is lower by one order of magnitude than that for Neb-PK-1. This disparity in threshold dose strengthens the argument that Neb-PK-2 is indeed the primary pupariation factor.

Both Neb-PK-1 and Neb-PK-2 elicit similar effects on the motor patterns of pupariation behavior as other synthetic pyrokinins tested earlier; that is they accelerate the onset and extend the duration of the immobilization/retraction phase [20]. The observation that retraction of the anterior segments of PK-injected larvae is completed a relatively long time after C has been previously reported [16], but it has not yet been evaluated in terms of a functional association with the R motor pattern preceding the "functionless" contraction pattern "C". Our observations in this study thus indicate that the actual process of anterior retraction is (i) independent of longitudinal contraction caused by cuticular shrinkage, and (ii) is temporally associated with the "C" motor pattern, as it is during normal pupariation, when it represents a "prelude" to the C motor pattern that coincides with shrinkage of the cuticle. The fact that shrinkage of the cuticle alone is sufficient to form a normally shaped puparium in PK-injected larvae creates a paradoxical situation whereby the performance of the behavioral motor programs of pupariation is temporally dissociated from actual morphogenesis of the puparium.

Our comparison of the timing of the "C" patterns of PK-injected larvae with the visible contraction of the controls revealed that the "functionless" contraction pattern "C" is also accelerated by moderate doses of the pyrokinins, though not to the same extent as the immobilization pattern. It is worth mentioning that tanning of the cuticle of PK-injected larvae is temporally associated rather with "C" motor patterns than with puparial contraction caused by cuticle shrinkage. This observation implies that (i) the mechanism of shrinkage of the cuticle surface is independent of the process of its phenolic tanning, and (ii) the integument may express different receptors for the pupariation accelerating peptides.

In summary, the sensitivity of various components of pupariation to pyrokinins decreases in the following order: immobilization > cuticular shrinkage > motor program for anterior retraction ≅ motor program for longitudinal contraction ≅ tanning of the cuticle. The possibility that Neb-PK-1 could play a minor role in the puparition process cannot be ruled out, but its primary function in the flesh fly remains unclear. A subfamily of pyrokinins that share the C-terminal hexapeptide sequence of Neb-PK-1, WFGPRLa, have been previously associated with the initiation of the onset of egg diapause in the silkworm *Bombyx mori* [4,12], and of diapause break in *Heliothis virescens* [11]. Whether Neb-PK-1 is involved in the regulation of diapause in the flesh fly is unknown. Regardless, of the two natural pyrokinins of *N. bullata*, Neb-PK-2, a product of the *hugin* gene, seems to be a more likely candidate for the primary pupariation factor. These results supplement and further elaborate our earlier studies of the mode of action of pyrokinins on pupariation [18,20].

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